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Recombinant technology

Transient expression of bacterial gene fragments in eukaryotic cells: implications for CD8$^+$ T cell epitope analysis

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Abstract

CD8$^+$ T cells are potent effectors of acquired immunity against some viruses and intracellular bacterial pathogens. Antigens recognized by CD8$^+$ T cells are small, 8–9 amino acid peptides derived from proteins produced by the pathogen. These peptides are presented by MHC class I molecules on the surface of the infected cell. When characterizing the CD8$^+$ T cell response to a bacterial or viral pathogen, it is often necessary to express an antigenic protein in a eukaryotic host cell that is capable of processing and presenting peptide epitopes to antigen-specific CD8$^+$ T cells. We describe a system designed to transiently express bacterial polypeptides and MHC class I molecules in eukaryotic cells. Recognition of these peptide–MHC complexes stimulates TNF production by antigen-specific CD8$^+$ T cell lines. This system should be useful for analysis of CD8$^+$ T cell epitope-containing bacterial gene fragments when expression of the entire bacterial protein is detrimental to the eukaryotic cell, or when overexpression of the bacterial gene is detrimental to the bacterial cloning strain. Furthermore, this system can be used for the rapid mapping of CD8$^+$ T cell epitopes within a protein.

Keywords: CD8$^+$ T cells; Epitope mapping; Gene expression; Bacterial antigens

1. Introduction

CD8$^+$ T cells, which recognize pathogen-derived peptides in the context of MHC class I molecules, are important effectors of adaptive immunity against microbial infection. They mediate protective effects through the elaboration of effector mechanisms that include cytolysis of infected cells and production of cytokines such as IFN-γ and TNF which mobilize and activate the host response to infection (Harty and Bevan, 1999). CD8$^+$ T cells are critical in vaccine-induced immunity against certain viruses and play important roles in resistance to infection with several bacterial and protozoan intracellular pathogens (Kaufmann, 1993; Ahmed and Gray, 1996). Thus, effective vaccines against these microbes will likely require identification of antigens that contain CD8$^+$ T cell epitopes.

CD8$^+$ T cell epitopes consist of 8–9 amino acid peptides that are selected for presentation based on MHC class I allele specific binding motifs and their ability to survive proteolytic digestion in the host cell (Rammensee et al., 1993). Two pathways, based on the initial location of the antigen, exist for the

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presentation of antigens to $CD8^+$ T cells. The endogenous MHC class I presentation pathway initiates with cytosolic proteins, either self- or pathogen-derived, that are degraded to peptides by the proteosome (York and Rock, 1996). The resulting peptides are actively transported into the endoplasmic reticulum where they stabilize MHC class I molecules and are transported to the cell surface for presentation to $CD8^+$ T cells. In this fashion, $CD8^+$ T cells are alerted to the presence of intracellular infection. In addition, it has been shown that molecules without obvious access to the cytoplasm can be presented by MHC class I molecules in vivo, a phenomenon originally described as cross-priming (Bevan, 1976). Recent studies demonstrate that professional antigen presenting cells (APC) such as macrophages and dendritic cells may process and present exogenous antigens to $CD8^+$ T cells (Jondal et al., 1996). Multiple pathways for presentation of exogenous antigens have been elucidated from in vitro experiments. Although exogenous pathways of MHC class I presentation are efficient in priming $CD8^+$ T cell responses in vivo, they do not appear to suffice for the display of antigens in a fashion that results in protective immunity (Shen et al., 1998). This result suggests that identification of antigens that are processed and presented by the endogenous MHC class I antigen presentation pathway is critical in the successful design of vaccines (Harty and Bevan, 1999).

Vaccine strategies rely on identification of antigens and in some cases the precise epitope recognized by the protective $CD8^+$ T cells. In some cases, this information has been difficult to obtain. Expression of pathogen-derived antigens in eukaryotic cells can render these cells targets for $CD8^+$ T cells, but generally requires low frequency stable transfection approaches (Harty and Bevan, 1992). Similarly, insertion of pathogen sequences into viral delivery systems (vaccinia, Sindbis, adenovirus) is time consuming and complicated by restricted cellular tropism of some viral vectors and potential cytopathic effects (Jensen et al., 1997). In addition, these strategies cannot overcome the possibility that the pathogen-derived proteins may be toxic when expressed by eukaryotic cells, as has been shown with some bacterial gene products (Demuth et al., 1994).

In this manuscript we describe a vector system for characterization of $CD8^+$ T cell antigens from bacteria based on transient expression of gene fragments in highly transfectable eukaryotic cells such as COS-7 or 293T. The system can be used to rapidly screen candidate antigens or as a tool to pinpoint the location of a $CD8^+$ T cell epitope. This system is less susceptible to the potential toxic effects of intact bacterial proteins since the construct is designed for expression of gene fragments. In addition, the system is not limited by the specific MHC class I molecules of the host cell since any MHC class I molecule can be co-transfected with the gene fragment of interest. Finally, the same system can be used to generate stable, antigen expressing transfectants of various eukaryotic cells.

2. Methods

2.1. Bacterial strains and cell lines

DH5α or DH10B (Gibco) *Escherichia coli* strains were grown in Luria broth (LB) (Sambrook et al., 1989). Bacterial strains were electroporated using an Invitrogen electroporator according to manufacturer's instructions. All eukaryotic cell lines were maintained at 37°C in a humidified atmosphere of 7% CO_2. COS-7 cells (ATCC# CRL-1651), P1.PyT cells (P1.HTR cells stably transfected with the polyoma large T antigen) (Scott et al., 1992), and 293T cells (293 cells stably transfected with SV40 large T antigen) (DuBridge et al., 1987) were maintained in Dulbecco's modified eagle medium (DMEM) (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (FCS), and supplemented with 2 mM L-glutamine, 5 mM HEPES buffer, 50 μM 2-β-mercaptoethanol, 100 U/ml penicillin and streptomycin, and 50 μg/ml gentamycin sulfate. WEHI 164 clone 13 cells (Espevik and Nissen-Meyer, 1986) were cultured in RPMI 1640 (Gibco) containing 10% FCS (RP10) and supplements listed above. P13.4 (Carbone and Bevan, 1990) are P815 ($H\text{-}2^d$ MHC) cells stably transfected with a eukaryotic vector expressing *E. coli* β-galactosidase and were maintained in RP10 media containing 400 μg/ml G418 (Gibco).

$CD8^+$ T-cell lines (CTL) specific for *Listeria monocytogenes* (LM) listeriolysin O (LLO)(91–99) or LM p60 (217–225) in the context of $H\text{-}2K^d$ were generated from BALB/c ($H\text{-}2^d$ MHC) mice and

maintained as described (Harty and Pamer, 1995; Harty and Bevan, 1996). $CD8^+$ T-cell line 0805B is specific for the *E. coli* β-galactosidase 876–884 epitope in the context of H-$2L^d$ (Rammensee et al., 1989). The specificity of each line was tested routinely using standard ^{51}Cr release assays (Harty and Bevan, 1992) with peptide-coated target cells.

2.2. *Cloning of pcDNA3m.l, H-2K^d and H-2L^d MHC class I molecules, and bacterial antigens*

The polylinker from plasmid pcDNA3 (Invitrogen, Carlsbad, CA) was removed by restriction digestion with *Hind*III and *Xho*I. The polylinker was replaced with a *Hind*III, *Xho*I digested oligonucleotide constructed by hybridization of two primers: 5′-AGCTTACCATGGGATCCTAACTAACC-TAAC-3′ and 5′-TCGAGTTAGGTTAGTTAG-GATCCCATGGTA-3′. Digested products were gel-purified and ligated with T4 DNA ligase. Recombinant plasmids were electroporated into DH5α *E. coli*. Transformants were selected on Luria agar (Sambrook et al., 1989) containing ampicillin (100 μg/ml). Plasmid DNA was isolated using Qiagen Maxi plasmid preps (Qiagen, Valencia, CA).

Plasmids pLd3 and pKd3 were constructed by isolation of *Bam*HI fragments encoding L^d cDNA (Joly and Oldstone, 1991) or the K^d cDNA (Lalanne et al., 1983) and cloning into the *Bam*HI site of pcDNA3 (Invitrogen). Expression of the L^d cDNA in COS-7 cells was examined by flow cytometry. 4×10^6 COS-7 cells were suspended in 0.4 ml cytomix (Van den Hoff et al., 1992) in a 0.4-cm gap cuvette. Fifteen microgram pLd3 was added and the cells were electroporated (300 V, 1000 μF, infinite *R*) using an Invitrogen electroporator. After 48 h, flow cytometry was performed on transfectants as previously described (Harty and Bevan, 1996) using anti-L^d antibody 28-14-8 (ATCC# HB 27) and, as a secondary antibody, FITC-conjugated rat anti-mouse IgG (Sigma, St. Louis, MO). Expression of H-$2K^d$ was examined in transfected COS-7 cells by flow cytometry as described above using anti-H-$2K^d$ antibody SF1-1.1.1 (ATCC# HB 159) conjugated to FITC.

Plasmid pβgal760-991 was constructed by digestion of pCH110 (Pharmacia, Piscataway, NJ) with *Rsa*I, and isolation of the 692 bp fragment encoding amino acids 760–991 of the *E. coli* β-galactosidase protein. This fragment was ligated to plasmid pcDNA3m.l. that had been digested with *Bam*HI and the ends made blunt with T4 DNA polymerase. This cloning allowed the in-frame expression of the β-gal fragment using the start codon and stop codons present in the polylinker of pcDNA3m.l.

Plasmids pJTH7-3 and pJTH20-3 were generated by PCR amplification of a LM LLO containing plasmid (Pamer et al., 1991) using the same 5′ end primer — 5′-CCCGGGATCCACCATGAAAAAA-ATAATGCTAG-3′, and primers 5′-GGATCCGGA-TCCATTCTTTAGCGTAAAC-3′ (for pJTH7-3), 5′-GGATCCGGATCCTATTATTCCGAATTCGC-TTTTAC-3′ (for pJTH20-3) (the *Bam*HI site is underlined). The PCR products were digested with *Bam*HI, gel-purified, and cloned, in frame, into the *Bam*HI site of pcDNAm.l. Plasmid p60Δ5 was constructed by PCR amplification of the LM p60 gene (Harty and Pamer, 1995) with primers 5′-CGG-GGATCCACTCCAGTTGCACC-3′ and 5′-CGG-GGATCCTGGAGCTGCTTG-3′. The PCR product was digested with *Bam*HI, gel-purified, and cloned, in frame, into the *Bam*HI site of pcDNA3m.l. Plasmid pLLO3 was generated by cloning a *Bam*HI fragment containing the LM gene (Pamer et al., 1991) into the *Bam*HI site of pcDNA3.

A DNA library encoding fragments of the LM LLO gene was constructed by PCR amplification of the entire LLO gene with primers 5′-CCCGGGATC-CACCATGAAAAAAATAATGCTAG-3′, and 5′-TTATTCGATTGGATTATCTAC-3′ using pLLO3 as a template. The PCR products were purified using a Qiagen PCR purification kit (Qiagen) and aliquots were subjected to a time-course DNase I treatment (Sambrook et al., 1989). After analysis by agarose gel electrophoresis, those aliquots containing LLO gene fragments approximately 30–100 bp in length were combined, extracted with phenol/$CHCl_3$ and ethanol precipitated. The ends of the DNA fragments were made blunt with T4 DNA polymerase and Klenow, and were ligated to plasmid pcDNA3m.l. that had been digested with *Bam*HI and the ends made blunt with T4 DNA polymerase and Klenow. Plasmid DNA was isolated from 1.5 ml of recombinant bacteria using Qiagen Mini Spin Preps (Qiagen) and 10/50 μl plasmid DNA was used per 100 μl transfection media for COS-7 cell transfections.

2.3. Transfection conditions and TNF bioassay

P1.PyT cells were stably transfected by suspending 10^7 cells in 0.5 ml cytomix (Van den Hoff et al., 1992) in a 0.4-cm gap cuvette. Ten micrograms of each plasmid DNA was added and cells were electroporated with an Invitrogen electroporator (320 V, 100 μF, infinite *R*). After 48 h transfected cells were selected for by growth in DMEM-10 media containing 300 μg/ml zeocin and 800 μg/ml G418.

COS-7 cells were plated at 15,000 per well in a 96-well flat bottom plate and grown overnight. Cells were transfected using the DEAE–dextran chloroquine method (Seed and Aruffo, 1987) with approximately 100 ng of each plasmid DNA. After 48 h the media was removed and 1000–30,000 CTL were added in 150 μl RP10 for 24 h. The human epithelial cell line 293T was transfected using the $CaCl_2$ method (Graham and vander Eb, 1973).

The TNF bioassay was used as described (Espevik and Nissen-Meyer, 1986). Fifty microliters of the supernatant was removed from the transfected COS-7 (or 293T) cell/CTL wells and added to 30,000 WEHI 164 clone 13 cells (seeded in flat-bottom 96-well plates) in 50 μl RP10 supplemented with 2 μg/ml actinomysin D and 40 mM LiCl. WEHI 164 clone 13 cells are highly sensitive to killing by TNF. After overnight incubation, 10 μl of Alamar blue (Acumed, West Lake, OH) was added per well and plates were incubated 3–6 h at 37°C/7% CO_2 before determining the $O.D._{570}$ and $O.D._{600}$. The Alamar blue reagent is converted from a blue color to a pink color by respiration of live cells. The death of the WEHI clone 13 indicator cells is a relative measure of TNF production and was calculated using the following formula: $(O.D._{570} - O.D._{600}) - x$, where $x = |O.D._{570} - O.D._{600}|$ of a well in which no cells were added. Percent survival was determined relative to wells containing WEHI 164 clone 13 cells alone.

3. Results

3.1. Construction of pcDNA3m.l

A shuttle vector used for the expression of eukaryotic cDNA was modified to allow the expression of bacterial gene fragments in eukaryotic cells. Shuttle vector pcDNA3 (Invitrogen) contains enhancer–promoter sequences from the human cytomegalovirus for high level transcription followed by a multiple cloning region, polyadenylation signal and transcription termination sequences from the bovine growth hormone gene to enhance RNA stability. It also encodes an ampicillin resistance gene and ColE1 origin for selection and maintenance of the plasmid in *E. coli*. In addition, the vector contains the SV40 virus origin of replication allowing replication of the vector as an episome in COS-7 cells. Since bacterial gene fragments lack introns and exons, they are similar to eukaryotic cDNA except for the lack of conservation in their translation initiation signals. The polylinker region of pcDNA3 was modified to overcome these differences. A synthetic eukaryotic ribosome binding site (a partial Kozak sequence) and an ATG start codon were added to aid in ribosomal binding to the bacterial mRNA fragments. In addition, a *Bam*HI cloning site for insertion of bacterial DNA was included and translation stop codons in all three reading frames were added 3′ to the *Bam*H1 cloning site. The resulting plasmid was designated pcDNA3m.l. (Fig. 1).

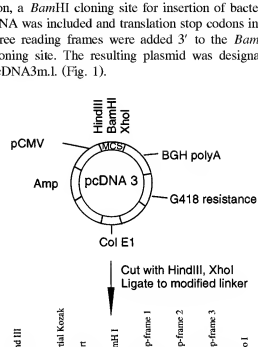


Fig. 1. Construction of pcDNA3m.l. Shuttle vector pcDNA3 (Invitrogen) was digested with *Hind*III and *Xho*I restriction enzymes and ligated to the indicated oligonucleotide containing *Hind*III and *Xho*I compatible ends, forming shuttle vector pcDNA3m.l.

3.2. Expression of a bacterial gene fragment and a murine MHC class I molecule in COS-7 cells

E. coli β-galactosidase (β-gal) was used as a model antigen to determine whether COS-7 cells (African green monkey kidney cells), a highly transfectable cell line, can transiently express bacterial gene fragments cloned into the modified pcDNA3 vector. The *E. coli* β-gal protein contains a $CD8^+$ T cell epitope (amino acids 876–884) that is presented by the murine H-$2L^d$ MHC class I molecule (Gavin et al., 1993). COS-7 cells transfected with a plasmid encoding H-$2L^d$ (plasmid pLd3) expressed the murine MHC class I protein as determined by flow cytometry using an H-$2L^d$-specific antibody (data not shown). When COS-7 cells were cotransfected with plasmid pLd3 and the modified pcDNA3 encoding amino acids 760–991 of β-gal (plasmid pβgal760–991), the transiently transfected COS-7 cells were able to stimulate specific production of TNF by $CD8^+$ T cells reactive with β-gal 876–884 (Fig. 2). The level of TNF produced was similar to that measured when COS-7 cells were transfected with plasmid pCH110, which encodes full length *E. coli* β-gal protein that has been optimized for expression in eukaryotic cells (not shown). In addition, P13.4 cells, an H-2^d MHC murine tumor cell line stably transfected with pCH110 (Carbone and Bevan, 1990), also stimulated similar levels of TNF by the β-gal 876–884 reactive $CD8^+$ T cells (data not shown). In contrast, COS-7 cells mock transfected, or transfected with plasmid pLd3 alone, did not stimulate TNF production by the β-gal-specific T cells (Fig. 2). Incubation of the pLd3/pβgal760-991 transfected COS-7 cells with $CD8^+$ T cells specific for different MHC-peptide complexes did not result in TNF production (not shown). These results indicate that pcDNA3m.l. directs the transient expression of a bacterial gene fragment in COS-7 cells, which are capable of processing and properly presenting the bacterial peptide in the context of a transiently transfected murine MHC class I molecule, in a manner that promotes antigen-specific TNF production by a murine $CD8^+$ T cell line.

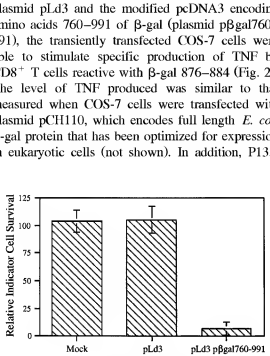


Fig. 2. COS-7 cells transfected with H2-L^d and a fragment of *E. coli* β-gal in pcDNA3m.l. stimulate TNF production from β-gal-specific $CD8^+$ T cells. TNF production was measured using a bioassay with supernatants from co-cultures of 1000 β-gal-specific CTL (Rammensee et al., 1989) and COS-7 cells, or COS-7 cells transfected with the indicated plasmids. Relative survival of indicator cells is inversely proportional to the amount of TNF detected. Data are presented as the mean ± S.D. of duplicate wells. All data are representative of at least three independent experiments.

3.3. Expression of fragments of a protein when the entire protein is toxic to the host cell

The *Listeria monocytogenes* (LM) p60 protein is a murein hydrolase that contains another well defined $CD8^+$ T cell epitope. The p60 epitope, spanning amino acids 217–225, is presented by the murine H-$2K^d$ MHC class I molecule (Pamer, 1994). During this study we attempted to clone the LM p60 gene into pcDNA3m.l. Although it was possible to isolate *E. coli* clones containing this plasmid on agar plates, they would not grow to high densities in liquid media as the cells would eventually lyse. This made the isolation of large amounts of plasmid DNA difficult. Presumably the LM p60 protein, a secreted hydrolase, was expressed and secreted into the periplasm of *E. coli*, causing the hydrolysis of the peptidoglycan and lysis of the bacterial culture. Therefore, a small 372 bp fragment encompassing the p60 $CD8^+$ T cell epitope was cloned into pcDNA3m.l., forming plasmid p60Δ5 and the expression of this construct was tested in this system. *E. coli* bearing this plasmid exhibited no lysis. Plasmid p60Δ5 and a plasmid encoding the murine H-$2K^d$ cDNA (plasmid pKd3) were used to cotransfect COS-7 cells, and presentation of the p60 217–225 peptide was probed with a K^d-restricted p60 217–225-specific $CD8^+$ T cell line. COS-7 cells were able to express, process and present the LM

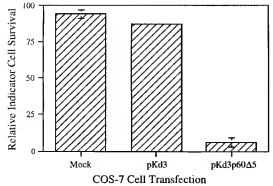


Fig. 3. COS-7 cells transfected with H-2K^d and a *L. monocytogenes* p60 gene fragment in pcDNA3m.l. stimulate TNF production from p60-specific CD8$^+$ T cells. COS-7 cells were either transfected with a plasmid encoding the murine H2-K^d MHC class I molecule alone (pKd3), or cotransfected with pKd3 and a plasmid encoding amino acids 146–265 of the LM p60 protein. After 48 h transfectants were incubated with 3000 LM p60-specific CD8$^+$ T cells. Twenty four hours later TNF in the supernatants was measured using a TNF bioassay. Data are presented as the mean ± S.D. of duplicate wells. All data are representative of at least three independent experiments.

p60 217–225 peptide in the context of the murine H2-K^d MHC class I molecule to stimulate TNF production by the p60 217–225 CD8$^+$ T cells (Fig. 3). Incubation of the transfected COS-7 cells with CD8$^+$ T cells specific for the LM LLO91–99 peptide (see below) did not result in TNF production (not shown). These results indicate that the pcDNA3m.l vector is useful for the expression of small, epitope-containing, bacterial fragments for CD8$^+$ T cell studies when cloning the entire bacterial protein is difficult or is detrimental to the bacterial host strain. Similarly, if expression of a certain bacterial protein in eukaryotic cells is toxic, as was described for the LM LLO protein (Demuth et al., 1994), this system may be used to express only a small fragment of that protein in eukaryotic host cells.

3.4. Epitope mapping using pcDNA3m.l

Even when a single protein is identified as the source of a CD8$^+$ T cell antigen, further work is required to identify the specific amino acid sequence within the protein which defines the epitope, or whether multiple epitopes are present (Sijts et al., 1996). Current techniques include synthesizing overlapping peptides covering the entire length of the protein, and testing each of these in peptide-pulsing experiments. This can be costly and time consuming. If the protein amino acid sequence and restriction element are known, peptides can be selected based on allele specific binding motifs (Pamer et al., 1991). However, this approach generally requires analysis of multiple peptides and will not identify those epitopes that do not fit the described motifs. It was of interest to determine whether the pcDNA3m.l. vector would be useful for mapping epitopes in our transient expression system.

The LM listeriolysin O (LLO) protein contains a well defined CD8$^+$ T cell epitope, LLO91–99. This epitope is also presented by the murine H2-K^d MHC class I molecule (Pamer et al., 1991). DNA encoding the entire LM LLO gene (pLLO3) or sequential deletions of the LLO gene were constructed and cloned into pcDNA3m.l. (Fig. 4A). These constructs

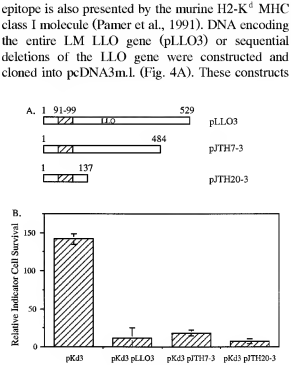
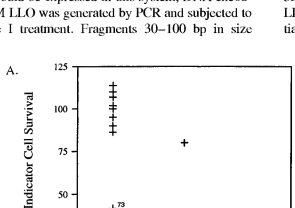


Fig. 4. Plasmid pcDNA3m.l. is useful for identifying fragments of a protein that contain a CD8$^+$ T cell epitope. (A) Regions of the LM LLO protein that were cloned into pcDNA3m.l. The numbers above the indicated fragments correspond to the amino acids of LLO, a 529 amino acid protein. The shaded portion of the fragment denotes the LLO91–99 epitope. (B) COS-7 cells were either transfected with the plasmid encoding the murine H2-K^d MHC class I molecule alone (pKd3), or cotransfected with pKd3 and the indicated LLO-expressing plasmids. After 48 h transfectants were incubated with 12,500 LLO 91–99-specific CD8$^+$ T cells, and 24 h later TNF in the supernatants was measured. Data are presented as the mean ± S.D. of duplicate wells. All data are representative of at least three independent experiments.

and pKd3 were used to contransfect COS-7 cells. Expression and presentation of the LLO91–99 epitope was probed with an H-2K^d-restricted LLO91–99-specific CD8$^+$ T cell line (Shen et al., 1998). As shown in Fig. 4B, each deleted construct stimulated TNF production by the LLO91–99-specific CD8$^+$ T cells. Incubation of the transfected COS-7 cells with CD8$^+$ T cells specific for the LM p60 217–225 peptide did not result in TNF production (not shown). The shortest LLO deletion plasmid tested, pJTH20-3, encodes a 137 amino acid peptide.

To determine whether even smaller fragments of LLO could be expressed in this system, DNA encoding LM LLO was generated by PCR and subjected to DNase I treatment. Fragments 30–100 bp in size were isolated and cloned into pcDNAm.I. Plasmid DNA was isolated from single bacterial colonies containing the recombinant plasmids and was used to cotransfect COS-7 cells along with plasmid pKd3. Two clones (#65 and #73) of 90 plasmids tested were able to stimulate TNF production by the LLO-specific CD8$^+$ T cell line (Fig. 5A). Restriction enzyme analysis of the plasmids revealed the inserts to be approximately 150 and 200 bp in size. The DNA sequence of the plasmid containing the smaller of the inserts revealed a 159 bp fragment in which several small LLO fragments had been fused (Fig. 5B). Only two of these fragments were in the proper LLO reading frame and could be considered potential epitope candidates, thus confining the epitope to



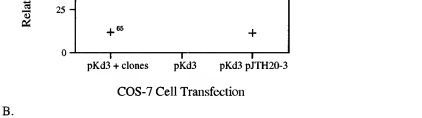


Fig. 5. Cloning small random fragments of LM LLO into pcDNA3m.I. can further define the region of DNA encoding a CD8$^+$ T cell epitope. (A) COS-7 cells were transfected with plasmid pKd3, encoding the murine H2-K^d MHC class I molecule, or cotransfected with pKd3 and plasmids containing DNase I fragments of the LM LLO gene cloned into pcDNA3m.I. (10 of the 90 clones tested are shown). After 48 h transfectants were incubated with 10,000 LLO 91–99-specific CD8$^+$ T cells, and 24 h later TNF in the supernatants was measured. Data are presented as the average of duplicate wells. The two positive clones, 65 and 73, are noted. (B) DNA sequence of positive clone 73 represents the fusion of several short stretches of the LM LLO sequence. The nucleotides of LM LLO are listed above the DNA sequence and the position of the translated LLO 91–99 epitope is shown by single letter amino acid code.

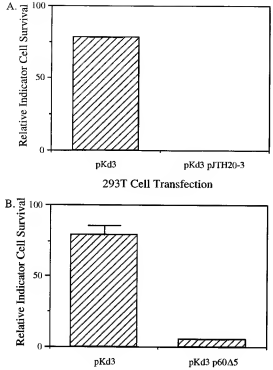


Fig. 6. 293T expression of known LM antigens cloned into pcDNA3m.l. stimulates TNF production by murine LM-specific CTL. (A) 293T cells were either transfected with a plasmid encoding the murine H-2K^d MHC class I molecule alone (pKd3), or cotransfected with pKd3 and a plasmid encoding amino acids 1–133 of the LM LLO protein. After 48 h transfectants were incubated with 12,500 LLO 91–99-specific CD8$^+$ T cells. After 24 h TNF in the supernatants was measured using a bioassay. (B) As in (A) except the LM p60 antigen encoding plasmid p60Δ5 was used in the cotransfection and transfectants were incubated with 3000 LM p60-specific CD8$^+$ T cells. Data are presented as the mean ± S.D. of duplicate wells. All data are representative of at least three independent experiments.

an 89 bp region. One fragment encoded the LLO 91–99 DNA sequence (nucleotides 442–469). The DNA sequence of positive clone #65 also encoded the LLO 91–99 sequence (not shown). These results indicate that this system should be useful for quickly identifying small fragments of a protein that contain a CD8$^+$ T cell epitope.

3.5. Expression of pcDNA3m.l.-encoded fragments in 293T cells

To ensure that the proper expression and processing of the pcDNA3m.l.-encoded bacterial gene fragments and murine MHC class I molecules were not unique to COS-7 cells, a cell line of a different origin was also tested in this system. The highly transfectable human renal epithelial cell line 293T was cotransfected with pKd3 and either pJTH20-3 or p60Δ5. After 48 h, the ability of the transfected 293T cells to stimulate TNF production by LLO91-99 or p60217-225-specific CD8$^+$ T cells was tested as described above. The transiently transfected 293T cells were able to stimulate specific TNF production by the LLO91-99 or p60217-225-specific CD8$^+$ T cells (Fig. 6A and B). The levels measured were similar to those using COS-7 cells, demonstrating that this transient expression system is not limited to a single cell type (see also Fig. 7).

3.6. Construction of a stably transfected cell line expressing a pcDNA3m.l.-encoded bacterial gene fragment

For analysis of CD8$^+$ T cell responses it may be necessary to construct stable cell lines that express an antigen of interest. The stable cell lines can be used as stimulators for the in vivo stimulation of antigen-specific CD8$^+$ T cell lines and clones. In addition, they can be used as target cells for deter-

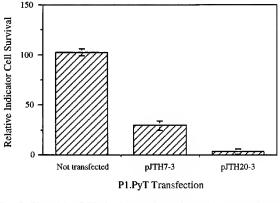


Fig. 7. Plasmid pcDNA3m.l. can direct the stable expression of bacterial gene fragments in a eukaryotic cell. The LM LLO91–99 encoding plasmids pJTH7-3 or pJTH20-3 (and a plasmid encoding H-2K^b) were electroporated into the H-2^d MHC murine tumor cell line P1.PyT and stably transfected cells were selected for by growth in G418 and zeocin. Transfectants were incubated with 30,000 LLO 91–99-specific CD8$^+$ T cells for 24 h, and TNF in the supernatants was measured using the TNF bioassay. Data are presented as the mean ± S.D. of duplicate wells. All data are representative of at least two independent experiments.

mining the specificity of a $CD8^+$ T cell line. To determine whether pcDNA3m.l. could direct the stable expression of epitope containing bacterial gene fragments, the murine tumor cell line P1.PyT was transfected with the LLO deletion constructs pJTH20-3 or pJTH7-3 and, for purposes not related to this study, a construct containing the murine $H\text{-}2K^b$ MHC class I cDNA. This plasmid, pKbzeo, encodes zeocin resistance. P1.PyT cells are derived from BALB/c mice ($H\text{-}2^d$ MHC) and express $H\text{-}2K^d$ MHC class I molecules. The pcDNA3m.l. construct encodes a selectable G418R marker (Fig. 1) and cells doubly transfected with the LLO deletion constructs and pKbzeo were selected by growth in G418 and zeocin containing media. Clones that grew out were tested for their ability to stimulate TNF^2 production by the LLO91–99-specific $CD8^+$ T cell line. Four out of twelve stable transfectants tested were able to stimulate TNF^2 production whether pJTH7-3, encoding a peptide of 484 amino acids, or the shorter LLO construct pJTH20-3, encoding a peptide of 137 amino acids, was expressed (Fig. 7). These results indicate that pcDNA3m.l. is also useful for the stable expression of bacterial fragments in eukaryotic cells for recognition by antigen-specific $CD8^+$ T cells. This could be beneficial for $CD8^+$ T cell studies when expression of an entire bacterial protein in eukaryotic cells is not successful.

4. Discussion

Much work has been focused on optimizing the expression of eukaryotic proteins in bacterial cells for industrial purposes, or to simplify systems in which to study a eukaryotic protein. Only recently has the need to express bacterial proteins in eukaryotic cells been realized. One such use is the expression of bacterial genes for DNA vaccination against bacterial pathogens (Uchijima et al., 1998). Another use involves characterization of bacterial proteins that stimulate T cells, specifically $CD8^+$ T cell responses. While it is apparent that eukaryotic cells can express bacterial DNA under control of a eukaryotic promoter, expression can be limited due to several factors. One factor may be codon usage differences between bacteria and higher eukaryotes (Nakamura et al., 1997). Another factor may be the toxicity of certain bacterial proteins in eukaryotic cells (Demuth et al., 1994). Since T cell epitopes consist of short peptides, a way to circumvent these difficulties would be to express fragments of the bacterial protein, that lack toxic activity.

We describe a system for the transient expression of bacterial gene fragments in eukaryotic cells. This system is similar to expression cloning systems developed previously by Boone and colleagues to express eukaryotic cDNA constructs in order to identify unknown human tumor $CD8^+$ T cell antigens (Brasseur et al., 1992; Brichard et al., 1993; Gaugler et al., 1994). In our study the cloning vector was modified in order to achieve efficient expression of bacterial gene fragments in eukaryotic cells. The vector contains a modified polylinker with a start codon in the context of an eukaryotic translation initiation consensus sequence to permit the appropriate initiation of translation. The promoter for expression of the bacterial fragment in eukaryotic cells is the potent CMV promoter. The pcDNA3m.l. vector is capable of driving the expression of several bacterial gene fragments derived from either the *E. coli* β-galactosidase or from the LM LLO and p60 proteins. Expression of these bacterial fragments and appropriate MHC class I molecules by COS-7 or 293T cells led to the stimulation of TNF production by antigen-specific $CD8^+$ T cells. Interestingly, bacterial expression of the intact p60 molecule, a secreted murein hydrolase, resulted in lysis of the host *E. coli* strain. In contrast, the shuttle vector containing the p60 gene fragment exhibited no toxicity in *E. coli* and efficiently stimulated p60-specific $CD8^+$ T cells after co-transfection of COS-7 cells with the $H\text{-}2K^d$ expressing construct. Thus, this system allows characterization of a bacterial-derived $CD8^+$ T cell epitope when the intact protein is toxic.

The ability to quickly identify unknown bacterial epitopes would greatly aid in the development of vaccines as well as contribute to the study of T cell responses against infection. Several techniques have emerged to quantify $CD8^+$ T cell responses in vivo, including the use of MHC class I/peptide tetrameric complexes for flow cytometry analysis (Altman et al., 1996). This powerful approach is dependent on the identification of the pathogen-derived peptide. Our results using sequential deletions, or DNase I digestion of the LM LLO gene, indicate that this

system can also be used to quickly identify the region of a protein that contains a $CD8^+$ T cell epitope.

A recent paper by Uchijima et al. (1998) indicates that in order to achieve effective $CD8^+$ T cell responses against an intracellular bacterium using DNA vaccination, it was necessary to optimize the codons of the bacterial epitope to those more frequently used by eukaryotic cells. If a small region of a bacterial protein that contains an epitope can be identified using this system, then fewer codons would need to be modified to increase the efficiency of the translation of the DNA vaccine.

One of the other impediments to $CD8^+$ T cell epitope characterization, particularly in human systems, is the polymorphic nature of MHC class I molecules and the lack of easily transfectable target cell lines that express the entire range of these MHC molecules. The transient transfection system we describe circumvents this problem because MHC class I molecules of interest can be co-transfected with the bacterial gene fragments, allowing the use of highly transfectable COS-7 or 293T cells as APC. In our experiments, we show that COS-7 cells can express 3/3 mouse MHC class I molecules, and are capable of processing and presenting three distinct bacterial antigens to activate $CD8^+$ T cell responses. In principle, this system could be adapted to any system where cDNA clones exist for the MHC class I molecules.

In summary, we describe a transient transfection system for characterization of $CD8^+$ T cell epitopes in bacterial gene fragments. The system is rapid, efficient and potentially adaptable to analysis of $CD8^+$ T cell responses from any mammalian species.

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